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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)*

79867800 1

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

"Tissue Repair"

5. Name of your agent *(if you have one)*

Murgitroyd & Company

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)*

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"Tissue Repair"

Field of the Invention

The present invention relates to compounds and methods of repairing tissue in diseases where the extracellular matrix is degraded. More particularly the invention relates to compounds including antibodies which increase extracellular matrix anabolism and the use of a novel pathway to find compounds which are capable of use in therapy to increase extracellular matrix anabolism.

Background

The Extracellular Matrix: Composition and Structure

The extracellular matrix (ECM) is a complex composite of proteins, glycoproteins and proteoglycans (PGs). Awareness of this complexity has been heightened by

the recognition that ECM components, individually or in concert with each other or other extracellular molecules, profoundly influence the biology of the cell and hence of the physiology of the whole structure in which the cell is embedded into. The functions of the ECM described so far are many but can be simply categorised as control of cell growth, providing structural support and physical stabilization, affecting cell differentiation, orchestrating development and tuning metabolic responses [30].

PGs are a family of heterogeneous and genetically unrelated molecules. The number of full-time as well as part-time members is constantly expanding. The term full-time and part-time refers to the fact that some known PGs can exist as glycoproteins and some proteins can be found in a glycosylated form. In general, PGs are composed of a core protein to which one or more GAG chains are covalently attached by N or O linkage. GAGs are highly anionic linear heteropolysaccharides made of a disaccharide repeat sequences [38]. However, there have been reports of PGs devoid of the GAG side chain [3;80]. GAGs can be classified into four distinct categories based on their chemical composition [38]. The first category is the chondroitin/dermatan sulphate (CS/DS) chain consisting of alternating galactosamine and glucuronic/iduronic acid units. A second class, which

is by far the most structurally diverse, is the heparin/heparan sulphate (H/HS) group which is composed of alternating glucosamine and glucuronic/iduronic repeats. The third type is the glucosamine and galactose containing keratan sulphate (KS) GAG. Hyaluronic acid (HA) is composed of glucosamine and glucuronic acid repeats. It is the most distinct GAG since it is not sulphated and is not covalently linked to the core protein of PG. Instead, HA binding to the PG core protein is mediated by a class of proteins known as HA binding proteins which exist in the ECM, on the cell surface and intracellularly [69].

Perlecan is a large HSPG with a core protein size of 400-450 kDa known to possess three HS chains. It was first isolated by Hassell et al. [32]. It acquired its name from its appearance in rotary shadowing electron microscopy where it looks like a pearl on a string. It is a large multi-domain protein and thus one of the most complex gene products [17;37]. Domain I is the N-terminus and contains acidic amino acid residues which facilitate the polymerisation of heparan sulphate [37]. However, recombinant domain I has been shown to accept either HS or CS chains; an observation that has been confirmed by *in vitro* study characterizing PGs synthesized in response to transforming growth factor β (TGF-β) and fetal calf serum showing that perlecan can be synthesized with

CS chains [9]. Ettner et al. [20] have shown that the ECM glycoprotein laminin binds to perlecan domain I, as well as domain V both of which can carry the HS side chain. Loss of the HS chain abolished the binding.

Globular domain II was postulated to mediate ligand binding by the low-density lipoprotein (LDL) receptor due to their homology [23;58]. Heparitinase treatment abrogates this interaction pointing to the fact that the HS GAG side chains are involved in the binding [23].

Domain III of perlecan contains an RGD tripeptide sequence that provides a binding capacity for integrin receptors and provides anchorage for the cell [13]. Yamagata et al. have shown using double-immunofluorescence that perlecan colocalizes with integrins in cultured fibroblasts [78]. This domain has also been shown to be homologous to the laminin short arm [36].

Domain IV is the largest domain of perlecan containing a series of Ig-like repeats similar to those found in the Ig superfamily of adhesion molecules leading to the speculation that it may function in intermolecular interactions [34]. Finally, domain V possessing three globular domains homologous to the long arm of laminin is thought to

be responsible for self-assembly and laminin mediated cell adhesion [10].

The multiplicity and variety of perlecan's structural domains are indicative of its potential functions. Perlecan, in addition to binding to laminin and integrins, has been shown to bind fibronectin via its core protein [36]. The HS chains of perlecan have also a very important functional role which has proven to be diverse. It has been reported that perlecan mediates the interaction between skeletal muscle cells and collagen IV via the HS GAG side chain [74]. Recent studies have led to the identification and characterization of perlecan as a ligand for L-selectin in the kidney [49]. Whether this interaction is via the core protein and/or the HS side chain is not clear. The group of Varki has identified in a series of experiments the HS GAG as well as heparin from endothelial cells as a ligand for both L- and P- selectins but not E-selectins [44;59]. The HS side chains in general, and those attached to perlecan core protein in particular, are known to bind growth factors such as fibroblast growth factors (FGF)-2, FGF-7, TGF-β, platelet factor-4 and platelet-derived growth factor-BB (PDGF-BB) [24;37]. The functional significance of these interactions has been highlighted by numerous studies demonstrating the role of perlecan in angiogenesis [4;65], the control of smooth muscle cell growth [8]

and the maturation and maintenance of basement membranes [14]. The functional importance of perlecan has been demonstrated by a study of mice lacking perlecan gene expression [14]. Homozygous null mice died between embryonic days 10 and 12. The basement membranes normally subjected to increased mechanical stresses such as the myocardium lost their integrity and as a result small clefts formed in the cardiac muscle leading to bleeding in the pericardial sac and cardiac arrest. The homozygotes also had severe cartilage defects characterised by chondrodysplasia despite that fact that it is a tissue which normally lacks basement membrane. This finding was interpreted as a potential proteolysis-protective function for perlecan in cartilage [14]. The delay in detecting abnormalities till E10 suggests a certain redundancy with compensatory molecules being able to substitute for perlecan such as the basement membrane HSPGs collagen XVIII [29] and agrin [28].

Large aggregating PGs are, to date, composed of four members; versican, aggrecan, neurocan and brevican [37]. The hallmark of these PGs is the ability to bind hyaluronic acid forming highly hydrated aggregates. They are also characterized by their tridomain structure composed of an N-terminal domain where HA binding occurs, a central domain carrying the GAG side chains and lectin binding C-terminus.

Versican is a PG with a core protein of 265 - 370 kDa which was originally isolated from human fibroblasts and is the homologue of the avian PG-M [84]. It can possess 10-30 chains of CS and has been also reported to carry KS GAG chains [83]. It is expressed by keratinocytes, smooth muscle cells of the vessels, brain and mesengial cells of the kidney. The N-terminal domain is responsible for the hyaluronic acid binding properties of versican [46]. The central domain of versican consists of the GAG binding subdomains, GAG-α and GAG-β. These subdomains are encoded by two alternatively spliced exons and this gives rise to different versican isoforms. To date four isoforms have been recognized. V0 contains both GAG-α and GAG-β. V1 and V2 are known to possess domain GAG-β and GAG-α respectively [83]. V3 is the variant which contains neither of the two subdomains and hence carries no CS/DS GAG side chains and has been localized in various mammalian tissues [48;60;79]. The third domain of versican is the C-terminus and consists of a lectin-binding domain, an EGF-like domain and a complement regulatory protein-like domain. This C-terminus binds the ECM glycoprotein, tenascin [2], heparin and heparan sulphate [66] and fibulin [1]. Versican is known to have an inhibitory effect on mesenchymal chondrogenesis [82], promotes proliferation [81] and migration via the formation of pericellular matrices

via its interaction with cell surface bound hyaluronic acid [21]. The formation of pericellular matrices is not only achieved via the core protein association with HA but also through GAG side chain interaction with the cytoskeletal associated cell surface receptor, CD44 [40]. The postulated role of versican in migration has been also further reinforced by the recent findings of its interaction with both L- and P- selectins via the CS/DS side GAG chains [41]. Furthermore, versican GAG side chains modulate chemokine response [33] and has been recently reported to possess growth factor binding capacity [85] and binding to β_1 integrin [77].

Aggrecan is another large aggregating proteoglycan. It is known to be a major structural component of cartilage. It is composed of three globular domains and two GAG attachment domains [75]. The N- terminal globular domain (G1) binds HA and link protein to form large aggregates. The second globular (G2) domain is unique to aggrecan and has no HA binding capacity. The function of this domain has not been clearly defined. The interglobular domain between the G1 and G2 contains proteolytic cleavage sites for metalloproteinases and thus been heavily investigated in pathologies where degradation of this domain is a hallmark, such as osteoarthritis. A KS domain is located at the C-terminus of the G2 domain followed by the CS domain. The CS domain is the largest domain

of aggrecan and the domain which contributes to the hydrated gel-like forming capacity of aggrecan and thus its importance in load-bearing function. The last domain is the globular domain (G3) which contains three modules: an epidermal growth factor-like domain, a lectin module and a complement regulatory module. This domain is responsible for the interaction of aggrecan with the ECM glycoprotein, tenascin.

Functions of Extracellular Matrix Proteoglycans

In addition to contributing to the mechanical properties of connective tissues, ECM PGs have biological functions which are achieved via specific classes of surface receptors. The two main, elaborately described, classes are syndecan and integrin receptor families [30]. However, other receptors have also been described to bind ECM components such as the selecting family of glycoproteins [59], CD44 with all its variants [25], cell surface enzymes such as hyaluronic acid synthases [67], and PGs [37]. It is important not to ignore the fact that the effects of the ECM do not and cannot, in an *in vivo* milieu, ever occur without the influence of other molecules. This statement is based on two well-described concepts. The first being that part of the effects of growth factors, cytokines, hormones and vitamins, as well as cell-to-

cell contact and physical forces is alteration of the ECM production. The second concept is that the effects of the ECM on the cell bear a striking similarity to those effects observed in response to the above mentioned factors. This is a phenomenon known as "mutual reciprocity" [30] which is an oversimplified view of a complex set of modular interactions, i.e. as defined by Hartwell et al. [31] "cellular functions carried out by "modules" made up of many species of interacting molecules". The outcome is a summation of all these modules which often interact with each other in a non-vectorial manner.

Integrins are a family of α,β heterodimeric receptors that mediate dynamic linkages between extracellular adhesion molecules and the intracellular actin cytoskeleton. Although integrins are expressed by all multicellular animals, their diversity varies widely among species [35;56;70]. To date 19 α and 8 β subunit genes encode polypeptides that combine to form 25 different receptors. Integrins have been the subject of extensive research investigating the molecular and cellular basis of integrin function. Integrins are major contributors to both the maintenance of tissue integrity and the promotion of cellular migration. Integrin-ligand interactions provide physical support for cell cohesion, generation of traction forces in cellular movement, and organise signalling complexes

to modulate cellular functions such as differentiation and cell fate. PGs are key ECM components which interact with integrins modifying their function and integrins, in turn, are key regulators of ECM PGs.

Currently little is known about the mechanisms underlying tissue organisation and cellular trafficking, and the regulation of those processes in disease, as well as determining the molecular basis of integrin function. No information has been provided to identify the function of distinct regions within the receptor.

Maintenance of the Extracellular Matrix

ECM homeostasis is maintained under normal physiological conditions by a fine balance between degradation and synthesis orchestrated by matrix metalloproteinase (MMPs) and tissue inhibitors of metalloproteinase (TIMPs). This homeostasis is critical in many physiological processes such as embryonic development, bone growth, nerve outgrowth, ovulation, uterine involution, and wound healing. MMPs also have a prominent role in pathological processes such as arthritis [50;53;62], chronic obstructive pulmonary disease [12;68] and atherosclerosis [51]. However, little is known about how they are anchored outside the cell.

Extracellular Matrix Catabolism and Anabolism

The ECM provides structural support as well as biological signals to almost every organ in the body. Of those organ systems involving ECM, is the lung where it provides structural support and acts as adhesive as well as a guiding cue for diverse biological processes. Collagens are the most abundant ECM component in the lung constituting 60-70 % of lung interstitium followed by elastin and PGs and glycoproteins [72].

The ECM composition of organs varies between the different anatomical and structural sites.

Lung PGs have just recently begun to be characterised. Perlecan and what is thought to be bamacan have been found in all lung basement membranes [15;55]. Of the SLR-PGs, lumican has been shown to be predominant and mainly found in the ECM of vessel walls and to a lesser extent in airway walls and alveolar septa [16]. Immunohistochemical studies have demonstrated the presence of biglycan in the peripheral lung, though in very small quantities, where it is associated with airway and blood vessel walls [7;16;18]. Furthermore, biglycan was shown to be associated with the epithelial cell layer particularly during development. Decorin has been

localized to the tracheal cartilage, surrounding blood vessels and airways, and interlobular septae [7]. However, Western analyses have demonstrated that decorin expression in the lung parenchyma is undetectable [16]. Similarly, it was shown in this study that fibromodulin expression is also undetectable; an observation confirmed by the undetectable mRNA levels for this PG by Westergren-Thorsson et. al. [76]. The large aggregating PG, aggrecan, is only found in tracheal cartilage associated with HA in a complex stabilized by the link protein [63]. On the other hand, versican can be found in small quantities in the airway and blood vessel walls [22], associated with smooth muscle cells [73] and fibroblasts [39], and has been co-localized with elastin fibers [63]. HA can be found in tracheal cartilage [63], basolateral surfaces of the bronchiolar epithelium and the adventitia of blood vessels and airways [26;27]. The HA receptor, CD44, is expressed mainly by airway epithelium and alveolar macrophages [42;47]. Syndecans have been reported to be heavily expressed by alveolar epithelial cells [52].

The Importance of the Extracellular Matrix in Disease Paradigm: Chronic Obstructive Pulmonary Disease: a major health problem

Awareness of extracellular matrix importance has been heightened by the recognition that it profoundly influences the biology of the cell and hence, both mechanically and biochemically, the physiology of the whole structure in which the cell is embedded. Interest in this arena not only transcends from curiosity driven science but from applied medical research. There may be a real lead to the development of a novel therapeutic intervention where part of the clinical presentation is precipitated by an imbalance in catabolism vs anabolism such as may be found in chronic obstructive pulmonary disease.

Chronic Obstructive Pulmonary Disease (COPD), comprising chronic bronchitis and emphysema, is a major cause of chronic morbidity and mortality throughout the world. In the UK, COPD is the fifth leading cause of death, causing 26,000 deaths and 240,000 hospital admissions annually. The cost to the NHS UK of COPD-related hospital admissions is in excess of £486 million annually [11]. Further costs are incurred due to co-morbidity such as respiratory infections and depression. Research into emphysema pathology and its treatment has been largely neglected because of the view that it is mainly self-inflected. Therefore strategies to effectively manage emphysema are needed in parallel to health promotion.

The Pathology of COPD

COPD is characterised by a progressive and irreversible airflow limitation [71] as a result of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema). Destruction of lung parenchyma is characterised by loss of alveolar attachments to the small airways, decreased lung elastic recoil and as a consequence diminished ability of the airways to remain open during expiration [6].

Although the main risk factor for COPD is tobacco smoking, other predisposing factors have been identified [64]. Emphysema is caused by inflammation, an imbalance of proteinases and antiproteinases in the lung (typified by hereditary α-1 antitrypsin deficiency) and oxidative stress which leads to the destruction of the ECM.

Current Treatments for COPD and Emphysema

To date, the only available drug treatments for COPD sufferers have focussed primarily on bronchodilation using anticholinergics and dual β2-dopamine2 receptor antagonists. Since the inflammation in COPD is resistant to corticosteroids, there is much anticipation as to the possible therapeutic opportunities of novel anti-inflammatory agents currently in development, which include phosphodiesterase inhibitors, nuclear factor KB

inhibitors and p38 MAP kinase inhibitors. Metalloproteinase (MMP) inhibitors are also currently being developed although in their current formulation serious toxic side effect are almost certain to limit their use. Retinoids have also been shown to induce alveolar repair though this remain largely disputed. However, notwithstanding all such hopeful activities, what is clearly lacking is an agent which may aid in the repair of injured ECM.

In summary, COPD/emphysema is a paradigm for diseases which have a strong element of ECM remodelling as a major contributor in their pathophysiology. Other organs which require tissue repair include but not exclusively skin, central nervous system, liver, kidney, cardiovascular system, bone and cartilage. Furthermore, current therapeutics have focused primarily on preventative or symptom-relieving treatments. However, due to the progressive nature of both diseases together with often late diagnosis, regaining normal function remains a problem.

Recently, novel therapeutic approaches targeting integrin function have been adopted. Very late antigen-4 (VLA4) or α4 integrin antagonists are currently in advance stages of trials for the treatment of asthma, multiple sclerosis and crohn's disease [43;45;54]. Antagonists to αvβ3 integrin have attenuated adjuvant-induced arthritis and now are

undergoing trials [5]. The target of the functional blocking or antagonism is attenuating inflammation and this has not been demonstrated to affect the ECM alteration usually associated with those conditions.

Description of the Invention

Accordingly, it is an object of the present invention to provide a compound for use in ECM anabolism. It is a further aim of the present invention to provide a technique to screen compounds for use in ECM anabolism.

According to the present invention there is provided a compound for use in tissue repair wherein the compound modifies the function of β1 integrin.

Modification includes a change in the function of, or the inhibition of the binding of, or the shedding of the β1 integrin.

Preferably the compound is an inhibitor of the β1 integrin.

More preferably the compound functionally blocks β1 integrin.

In one embodiment the compound binds the molecule in the region of amino acid residues 82-87. It is to be

understood, however, that this is not limiting and there are other domains in the β1 integrin molecule to which the compound binds.

In the known sequence, residues 82-87 are considered to be the residues of the sequence identified by the nomenclature SEQ ID NO 1: nprgsk (Asparagine-Proline-Arginine-Glycine-Serine-Lysine).

In a further embodiment the compound is a peptide or a chemical or an analogue thereof. Preferably the compound is a synthetic peptide or a synthetic chemical.

Analogues of, and for use in, the invention as defined herein means a peptide modified by varying the amino acid sequence e.g. by manipulation of the nucleic acid encoding the protein or by altering the protein itself. Such derivatives of the amino acid sequence may involve insertion, addition, deletion and/or substitution of one or more amino acids

Preferably such analogues involve the insertion, addition, deletion and/or substitution of 5 or fewer, and most preferably of only 1 or 2 amino acids.

Analogues also include derivatives of the defined peptides, including the peptide being linked to a coupling partner, e.g. an effector molecule, a label,

a drug, a toxin and/or a carrier or transport molecule. Techniques for coupling the peptides of the invention to both peptidyl and non-peptidyl coupling partners are well known in the art.

In a further embodiment the compound is an antibody.

The antibody should preferably be a humanised antibody.

Alternatively the antibody could be a chimeric antibody.

Alternatively the antibody could be a human antibody.

In one embodiment the antibody could be based on or derived from the functional modifying antibody of β1 integrin obtainable as a commercial clone JB1a from Chemicon.

In a further embodiment the antibody could be based on or derived from the antibody 6S6. 6sS targets a domain of the β1 integrin yet to be specifically identified, but thought to be in the EGF-like repeat domain distinct from the 82-87 domain targeted by the JB1a antibody.

An "antibody" is an immunoglobulin, whether natural or partly or wholly synthetically produced. The term

also covers any polypeptide, protein or peptide having a binding domain that is, or is homologous to, an antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies are the immunoglobulin isotypes and their isotypic subclasses and fragments which comprise an antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies.

The binding member of the invention may be an antibody such as a monoclonal or polyclonal antibody, or a fragment thereof. The constant region of the antibody may be of any class including, but not limited to, human classes IgG, IgA, IgM, IgD and IgE. The antibody may belong to any sub class e.g. IgG1, IgG2, IgG3 and IgG4.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any binding member or substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin-binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are

therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can perform the function of antigen binding.

Examples of such binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. *et al. Nature* 341:544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird *et al. Science* 242:423-426 (1988); Huston *et al. PNAS USA* 85:5879-5883 (1988)); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Hollinger *et al. PNAS* 90:6444-6448 (1993)).

Antibodies according to the invention can be prepared according to standard techniques. Procedures for

immunising animals, e. g. mice with proteins and selection of hybridomas producing immunogen specific monoclonal antibodies are well known in the art. The antibody is preferably a monoclonal antibody.

In one embodiment, the present invention provides a compound for use in tissue repair in the lung, skin, liver, kidney, nervous system, cartilage, bone and cardiovascular system.

A further aspect of the invention provides a method to screen for compounds for use in tissue repair, the method including the step of determining the ability of a compound to modify the function of the β1 integrin.

Modification includes a change in the function of , or the inhibition of the binding of, or the shedding of the β1 integrin.

Preferably the method includes the step of determining the ability of a compound to bind the domain corresponding to residues 82-87 of β1 integrin (residues nprgsk (Asparagine-Proline-Arginine-Glycine-Serine-Lysine)).

A yet further aspect of the present invention provides a compound identified from the method described herein.

A yet further aspect of the present invention provides a medicament suitable for use in tissue repair wherein the medicament includes as an active ingredient, a compound which modifies the function of β1 integrin.

Preferably the medicament binds amino acid residues 82-87 of β1 integrin (residues nprgsk (Asparagine-Proline-Arginine-Glycine-Serine-Lysine)).

The medicament may be administered together with any suitable carrier.

The invention further provides the use of an antibody to β1 integrin in the preparation of a medicament for treatment of injured tissue administered via any therapeutic route.

It is thought that a compound according to the present invention may act by shedding the β1 integrin and/or affecting MMPs/TIMPs balance.

Substitutions may be made to the binding epitope as defined in the present invention, for example amino acid residues may be substituted with a residues of the same or similar chemical class, and which result in no substantial conformational change of the binding epitope.

In a yet further embodiment of the present invention, there is provided a compound for use in tissue repair wherein the compound binds to any nature-similar or mimetic molecule which has conformational homology to the β1 integrin. In other words the three-dimensional shape of the mimetic molecule is substantially super-imposable upon the three-dimensional shape of the β1 integrin.

Preferably the nature-similar or mimetic molecule has a conformational homology to amino acid residues 82-87 of the β1 integrin.

Residues 82-87 are known to have the sequence nprgsk (Asparagine-Proline-Arginine-Glycine-Serine-lysine)

Preferred features of each aspect of the invention are as for each other aspect, *mutatis mutandis*, unless the context demands otherwise.

The invention is exemplified herein with reference to the following non limiting examples which are provided for the purpose of illustration and are not to be construed as being limiting on the present invention. Further reference is made to the accompanying figures wherein;

Figure 1 illustrates dose- and time-dependent effects of functional modification of β1 integrin and neutralising TGF-β on ECM PG from H441 cell lines,

Figure 2 shows the presence of a 110kDa β1 integrin in the media of chondrocytes in alginate cultures and H441 cells separated onto 6% SDS-polyacrylamide gels following β1 integrin function modulation,

Figure 3 illustrates the time-dependent effect of functional modification of β1 integrin on ECM PGs in human lung explants and the lack of effect using a control β1 integrin antibody,

Figure 4 illustrates the effects of functional modification of β1 integrin on ECM PGs in human lung explants,

Figure 5 shows Western analyses demonstrating the increase in inactive MMP9 in the media of human lung explants following β1 integrin function modulation,

Figure 6 shows Western analyses demonstrating the increase in ECM PG, perlecan in the media of cultured human lung cells (Collagenase

digest alone or in co-culture with the Elastase digets) following β1 integrin function modulation (β1 Ab). The figure also shows the effect of cyclohexiimide (CXH) and APMA on the PG response to β1 integrin function modulation. In addition, the effect of neutralising MMP7 and 9 and MMPs are demonstrated,

Figure 7 shows Western analyses demonstrating the increase in TIMP1 in the media of cultured human lung cells (Collagenase digest alone or in co-culture with the Elastase digets) following β1 integrin function modulation (β1 Ab). The figure also shows the effect of cyclohexiimide (CXH) and APMA on the TIMP1 response to β1 integrin function modulation. In addition, the effect of neutralising MMP7 and 9 and MMPs are demonstrated.

Figure 8 shows Western analyses demonstrating the decrease in MMP1 in the media of cultured human lung cells (Collagenase digest alone or in co-culture with the Elastase digets) following β1 integrin function modulation (β1 Ab). The figure also shows the effect of cyclohexiimide (CXH) and APMA on the TIMP1 response to β1 integrin function modulation.

In addition, the effect of neutralising MMP7 and 9 and MMPs are demonstrated,

Figure 9 shows Western analyses demonstrating the increase in inactive MMP9 in the media of cultured human lung cells (Collagenase digest alone or in co-culture with the Elastase digests) following β1 integrin function modulation (β1 Ab). The figure also shows the effect of cycloheximide (CXH) and APMA on the TIMP1 response to β1 integrin function modulation. In addition, the effect of neutralising MMP7 and 9 and MMPs are demonstrated,

Figure 10 shows a photograph demonstrating the effect of β1 integrin functional modification on the size lungs of emphysematous mice (PPE),

Figure 11 shows haematoxilin and eosin staining of 5um formalin-fixed paraffin embedded section demonstrating the effect of β1 integrin functional modification on repair of lung architecture in elastase-induced emphysema in mice,

Figure 12 demonstrates the effect of β1 integrin functional modification on air space

enlargement in Elastase induced emphysema in mice,

Figure 13 demonstrates the effect of β1 integrin functional modification on active TGFβ1 levels in the bronchoalveolar lavage fluid in Elastase induced emphysema in mice,

Figure 14 demonstrates the correlation of active TGFβ1 levels in the bronchoalveolar lavage fluid and air space enlargement index and the effect of β1 integrin functional modification in Elastase induced emphysema in mice,

Figure 15 shows Western analyses demonstrating the increase in ECM PG, perlecan in the media of cultured human lung cells (NCI-H441) following β1 integrin function modulation (β1 Ab). 6S6 anti β1 integrin antibody was also used. The figure also shows the effect of cycloheximide (CXH) and APMA on the PG response to β1 integrin function modulation, and

Figure 16 shows Western analyses demonstrating the increase in inactive MMP9 in the media of cultured human lung cells (NCI-H441) following

β1 integrin function modulation (β1 Ab). 6S6 anti β1 integrin antibody was also used. The figure also shows the effect of cycloheximide (CXH) and APMA on the PG response to β1 integrin function modulation.

In a preliminary experiment, the present inventors attempted to investigate the role of the cell surface receptors in the synthesis of ECM which are altered in diseases such as COPD and are important for lung and cartilage function microscopically and macroscopically. The importance of those ECM molecules in health and disease is not exclusive to the lung.

The results described herein demonstrate that functional modification of β1 integrin through a domain corresponding to amino acid residues 82 to 87 and to a lesser extent through a domain not yet specifically identified, but thought to be in the EFG-like repeat domain distinct from the 82 to 87 domain, induces a substantial time- and dose-dependent increase in ECM in a human lung epithelial cell line (NCI-H441) in monolayer and human lung explants as well as human lung derived culture in monolayer or co-culture system. The response was observed using two different antibodies against β1 integrin though the magnitude of the response was variable. These domains are different from those

previously described which bind to the amino acid sequence residues 207 to 218. It is also distinct from the known stimulatory domains which are localised to those amino acid residues and residues 657 to 670 and 671 to 703. Modulation of the cytokine TGF-β induced a less profound increase which was also time- and dose-dependent. This increase in all ECM PGs was sustained for extended periods of time without any additive doses.

These experiments demonstrate a novel finding which is that an increase in ECM can be achieved via the modulation of cell surface receptors and to a much lesser extent by modulating the binding of a soluble factor in a time- and dose-dependent manner in pulmonary derived cells and tissues in animal models. One potential, but non-binding mechanistic hypothesis is that this modulation may have led to alteration in the proteinase/antiproteinase balance which can be sequestered onto the surface of cells. Furthermore, the response could be a result of changes in gene transcription or translation. Experiments have demonstrated that the response is due to combination of both of the above. The ECM response to β1 integrin functional modification was accompanied by an increase in TIMP1, inactive MMP9 and active TGFβ1 and a decrease in MMP1.

When administered to animals which have emphysematous lungs, the treatment reversed the abnormal increase in the mean linear intercept (MLI) as an index of air space enlargement, lung size and signs of inflammation.

The potential of these findings lie in tissue repair in disease where the matrix is degraded and cannot be replenished as in diseases that include but not exclusive to COPD. The finding may offer a venue for therapeutic intervention in diseases where the only current lines of therapy focus on alleviating the symptoms by the use of anti-inflammatory agents but has no potential for regaining function. This could be achieved via the administration of humanised, chimeric or human antibodies or synthetic peptides or chemicals capable of binding β1 integrin.

In summary, the results herein address a different potential therapeutic modality which focuses on increasing ECM anabolism instead of decreasing catabolism.

Experimental Protocol

Human lung explants culture and human lung derived cell isolation

Human lung tissue specimens were obtained with consent and cultured as either 20-30mg explant strips or cells.

Cell were isolated by sequential digestions modified from methods by Murphy et.al and Elbert et. al. [19;57] where the tissue (10g) was washed using HEPES buffer (buffer A: 0.13M NaCl, 5.2mM KCl, 10.6mM Hepes, 2.6mM Na_2HPO_4, 10mM D-glucose, pH 7.4). The tissue was then incubated in 40 ml buffer A containing 0.855 mg Elastase (Roche) 0.5% trypsin, 200U/g DNAsI, 1.9mM $CaCl_2$, and 1.29mM $MgSO_4$ for 40 minutes at 37°C.

The digest buffer is then aspirated and suspended cells washed three times in buffer A. The cells between each wash were pelleted by centrifuging the suspension for 10 minutes at 1100rpm and 4°C. After the final was the cells were resuspended in buffer A, filtered through 40um filter and then subjected to discontinuous Percoll gradient (1.089/1.04g/ml). The cells were then plated onto multi-well culture plates and tissue culture transwells of 0.3um pore size(Sigma) and maintained in culture using 1:1 DMEM/F12:Small airway growth media (Cambrex BioScince Wokingham Ltd.) containing 1% foetal calf serum L-glutamine and antibiotic/antimycotic/antifungal mixture and maintained at 5% in an CO_2 incubator.

The remaining tissue was treated with DMEM containing 40% foetal calf serum to inactivate the digestive enzymes and then washed using solution A. The tissue was then incubated in DMEM containing 1mg/ml Collagenase, 0.5% trypsin and 200U/g DNAsI and maintained at 5% in an CO_2 incubator. The cell suspension was washed as above and cells seeded on multiwell culture plates and maintained in DMEM (Sigma Aldrich) containing 10% foetal calf serum, L-glutamine and antibiotic/antimycotic/antifungal mixture and maintained at 5% in an CO_2 incubator.

Adenocarcinoma cell line derived from the lung were also used (H441) to test the effect of the antibodies on matrix synthesis. This cell line has epithelial type II characteristics.

Cultures were subjected to serum starving overnight in a medium containing 0.5% foetal calf serum. Some collagense digested plated were co-culture with the Elastase digest transwells at the time of initiating the starvation.

Functional modifying antibody of β1 integrin (Chemicon, clone JB1a) was added to the cultures at concentration of 1.44 and 0.48 µg/ml. The β1 integrin stimulatory antibody TS2/16 was also added at 0.9 µg/ml for 1 hour to demonstrate the specificity of the JB1a action. The β1 integrin inhibitory antibody

6S6 was also added at 1 µg/ml and 2 µg/ml for 1 hour. TGFβ neutralising antibody (R&D systems, clone 1D11) was added at a concentration of 0.1 and 0.3 µg/ml where at the lower concentration it neutralises TGFβ isoforms 1 and 3 and isoform 2 at the higher concentration. After antibody addition to the cells in culture, the medium was aspirated and the cell layer rinsed twice with ice-cold PBS (calcium- and magnesium-free). The media was aspirated and preserved after the addition of protease inhibitors at -80°C. PGs were extracted from the cell layer by extraction buffer containing protease inhibitors (4M guanidium-HCl, 4% (w/v) CHAPS, 100mM sodium acetate buffer at pH 5.8 containing protease inhibitors) for 24 hours at 4°C.

In additional experiments, the effect of protein synthesis inhibition on β1 integrin mediated PG increase was tested by pretreating the human lung derived cells with 25uM cycloheximide.

The effect of non-specific activation of MMPs on β1 integrin mediated PG increase was tested by pretreating the human lung derived cells with 0.5M APMA (aminophenylmercuric acetate).

To investigate the involvement of selected MMPs in initiating the response observed with β1 integrin,

specific neutralising antibodies for MMP7 (1:1000, R&D systems) and MMP9 (1:1000 of clone 6-6B, Oncogene Research Products. A homophe-hydroxamic acid based broad spectrum inhibitor of MMPs was also used at 2.3nM (MMP inhibitor III, Calbiochem).

The total protein concentration was estimated using the Bradford method.

Sample Preparation for Composite Polyacrylamide-Agarose Gel Electrophoresis

The extracts were precipitated overnight with 9 v/v ethanol at -20°C, centrifuged at 12,000 rpm for 40minutes at 4°C then resuspended in 0.5M sodium acetate (pH 7.3) and precipitated again with ethanol overnight and centrifuged. Samples were resuspended in 0.5% SDS and mixed with 1:1 v/v with 50%w/w sucrose in 10mM Tris-HCl (pH 6.8), 0.5% SDS and 0.05% bromophenol blue. 20ug of protein was used for gel loading.

Gel electrophoresis

Composite gels (1.5mm thick) containing 0.6% agarose and 1.2% polyacrylamide in Tris-sodium acetate buffer (10mM, pH 6.8) containing 0.25mM sodium sulphate were used for the separation of large PG, versican,

aggrecan and perlecan, under associative conditions according to the method of Carney.

SDS-PAGE was also used to separate the denatured PG and proteins.

After electrophoretic separation, the samples were transferred onto Hybond ECL-nitrocellulose membrane (Amersham Pharmacia) using a wet blotting unit (BioRad). Membranes were blocked with 5% Milk in TBS pH 7.4 containing 0.1 % v/v Tween-20 and 0.1% sodium azide for 1 hours at room temperature and then incubated with primary antibodies diluted in TBS-Tween 20 for 1 hour at room temperature or overnight at 4°C.

The primary antibody for versican (12C5) was mouse anti-human at 1/500 dilution (Hybridoma Bank, Iowa City, Iowa). This antibody recognizes the hyaluronic acid binding domain of versican [61]. Aggrecan antibody was used at dilution of 1/500 aggrecan (Serotec, HAG7E1). Due to the fact that the exact epitope recognised by this antibody is unknown, additional antibodies were used. Perlcan antibody was used at a dilution of 1/1000 (7B5, Zymed Laboratories). This antibody has been demonstrated to be immunoreactive to non-degraded forms of perlecan [56]. MMP1 (41-1E5), inactive MMP9 (7-11C) and TIMP1

(7-6C1) antibodies were all from Oncogene Research Products and used at 1:1000 dilution.

Some blots were stripped using 100mM 2-mercaptoethanol, 2% SDS and 62.5mM Tris-HCl (pH 6.7) at 56°C for 20 minutes. They were then re-probed using a different antibody.

A horseradish peroxidase (HRP) labelled secondary antibody (goat anti mouse Ig, Dako) was added. Signal was visualised using the ECLplus (enhanced chemiluminescence) assay (Amersham Pharmacia).

The same analyses as detailed above were performed using extracts subjected to pre-clearing of the functional modifying antibodies by immunoprecipitation using protein A sepharose according to manufacturer's instructions (Amersham Pharmacia).

Immunohistochemistry

In additional experiments, immunohistochemical staining for PG was performed on 5 um thick frozen OCT-embedded sections from human lung explants. The slides were blocked by incubating with universal blocking solution for 10minutes at room temperature followed by biotin blocking solution for 10 minutes (Dako). Sections were then rinsed with TBS (0.5 M

Tris, pH 7.6, 1.5 M NaCl), and incubated with the primary antibody. After washing with TBS, the tissue was incubated with a 1/200 biotin-labeled goat anti-mouse in TBS for 1 hour, rinsed with TBS and then further incubated with 1/100 alkaline phosphatase-conjugated avidin in TBS for 1 hour. After further washing, sections were developed with Fast Red salt 1mg/ml in alkaline phosphatase substrate for 15 minutes at room temperature. Sections were counter-stained with Gil's Haematoxylin for 45 seconds, then washed with water. The sections were covered with a thin layer of crystal mount and dried in the oven at 37°C, overnight.

Therapeutic effect using an in vivo animal model of injury: Model of emphysema induced by instillation of porcine pancreatic elastase emphysema

Female C57/BL6 mice (6-8 weeks old) were instilled intra-tracheally using a metal cannula with 1 IU/g body weight porcine pancreatic elastase (Roche). Mice were sampled at day 10 post instillation and histology examined to verify the presence of air space enlargement. At day 12, mice were treated intra-tracheally with the integrin antibody at 50 ug/animal in sterile PBS. Control group was instilled initially with PBS and at day 12 with isotype control IgG1 (50ug/animal). At day 19 post elastase instillation, the animals were sacrificed,

bronchoalveolar lavage fluid (BALF) collected and used to quantify the cytokines (KC (murine homologue of human IL8) and active TGFb1) using sandwich ELISA (R & D Systems).

The lungs were then removed en bloc and formalin-fixed at a pressure of 25cm water, for histological assessment of damage and morphometric analysis (mean linear intercept). Blocks were sectioned at 5um thickness and stained using Haematoxylin and Eosin. Sagittal sections were used from each animal. Images from 10 fields per section at 100x magnification were digitised and analysed using Scion image (NIH). Actual field size was 1.33 (H) x 1.03 (V) mm. The number of alveolar walls intercepting a horizontal and a vertical line was counted. Mean linear intercept was calculated from each field (horizontal and vertical) by dividing the length of the line by the number of intercepts.

Our experiments demonstrate a novel finding which is that the an increase in ECM PGs anabolism can be achieved via functional modification of the cell surface β1 integrin and to a much lesser extent to neutralising TGFβ in both time- and dose-dependent manner in human lung explants and human lung derived cell co-cultures as well as pulmonary derived epithelial cell line. Our experiments have demonstrated that the increase in ECM PGs was

partially due to de novo protein synthesis. The changes were accompanied by an increase in TIMP1, inactivation of MMP9 and decrease in MMP1.

We have also induced emphysematous injury in the lung using 20 IU/animal of porcine pancreatic elastase. Elastase induced a statistically significant three fold increase in the mean linear intercept (MLI) accompanied by an increase in lung size. Emphysematous mice treated by a single intratracheal dose of anti β1 integrin at day 13 showed marked reduction in lung size at day 20. The change was accompanied by a significant reduction in the MLI.

Furthermore, porcine pancreatic elastase resulted in a decrease in active TGFβ1 in the bronchoalveolar lavage which appeared to be reversed by the treatment. The levels of active TGFβ1 exhibited a statistically significant correlation ($r=0.96$, $p<0.01$) with the MLI.

All documents referred to in this specification are herein incorporated by reference. Various modifications and variations to the described embodiments of the inventions will be apparent to those skilled in the art without departing from the scope of the invention. Although the invention has been described in connection with specific preferred embodiments; it should be understood that the

invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in the art are intended to be covered by the present invention.

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Figure 1. The effect of β1 integrin functional modification on proteoglycans in H441 cells

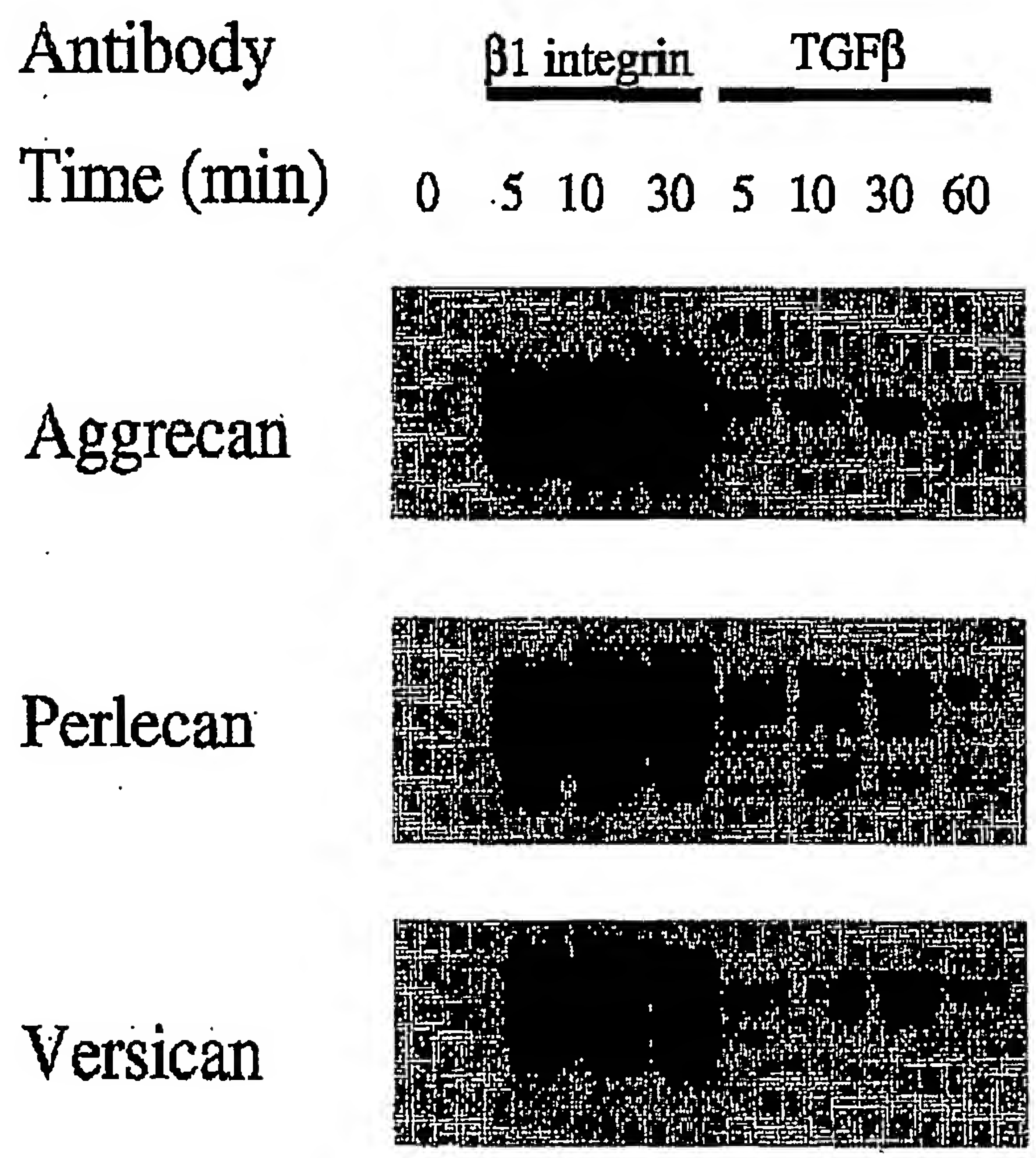


Figure 2.

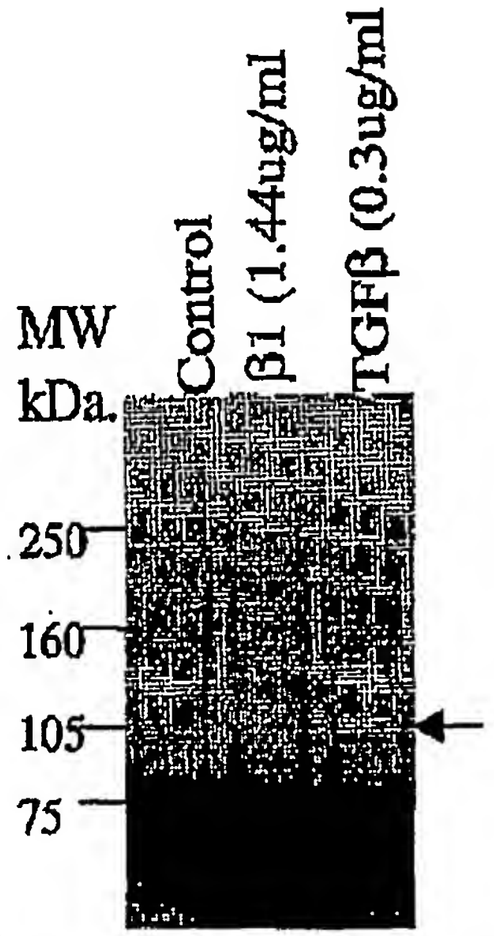


Figure 3.The effect of β1 functional modification on perlecan expression in human lung explants.

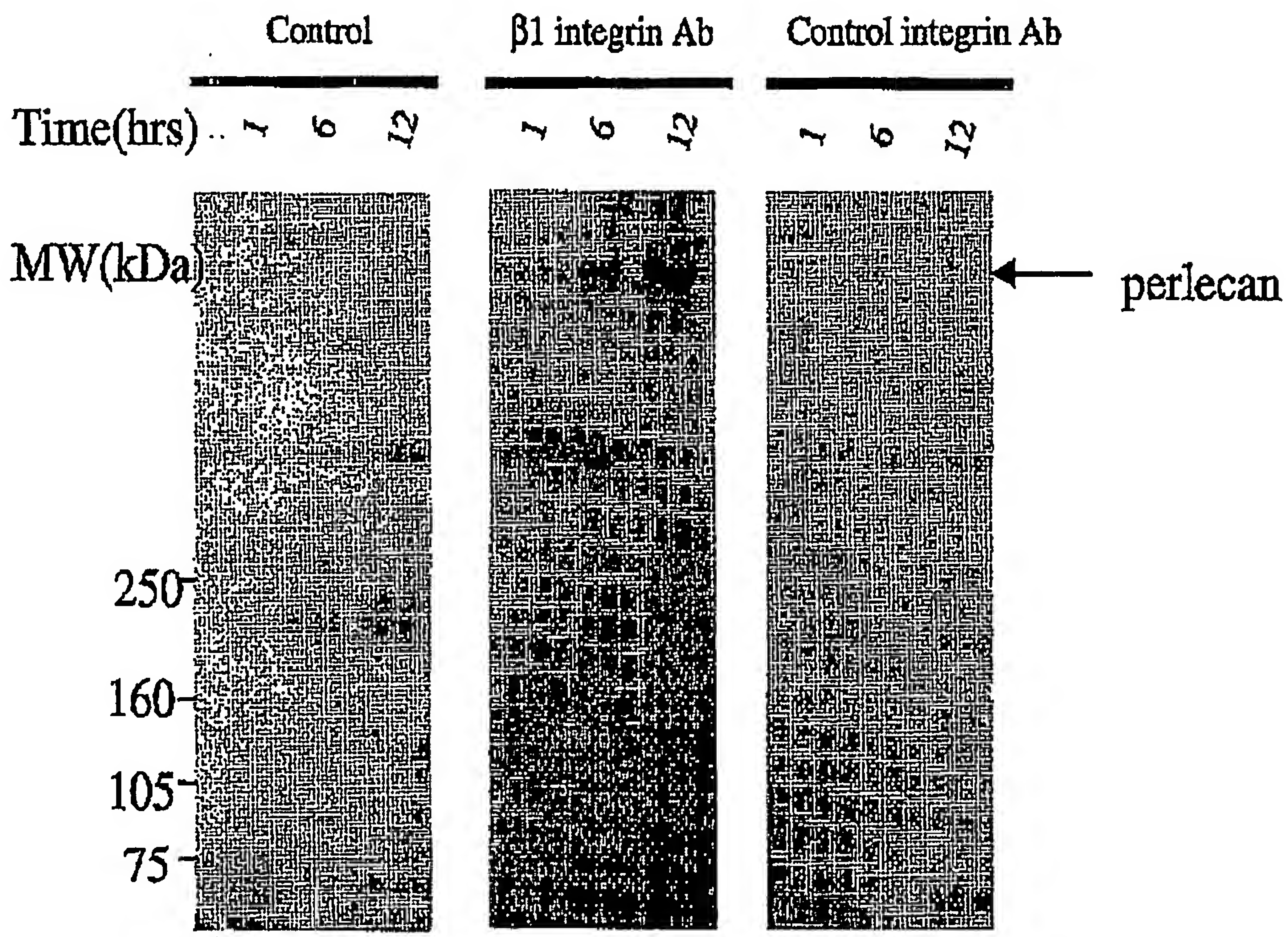


Figure 4. The effect of β1 functional modification on perlecan expression in human lung explants.

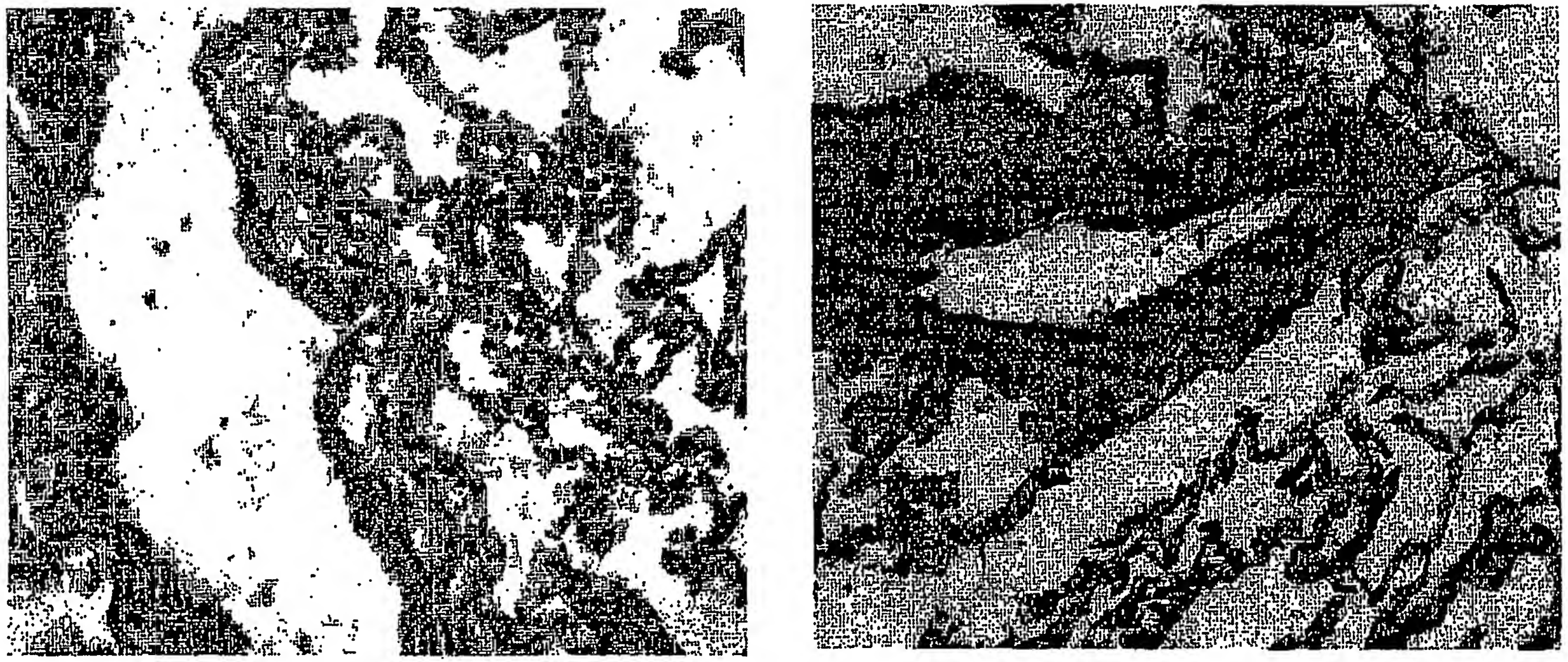


Figure 5. The effect of β1 integrin functional modification on MMP9 in human lung explants

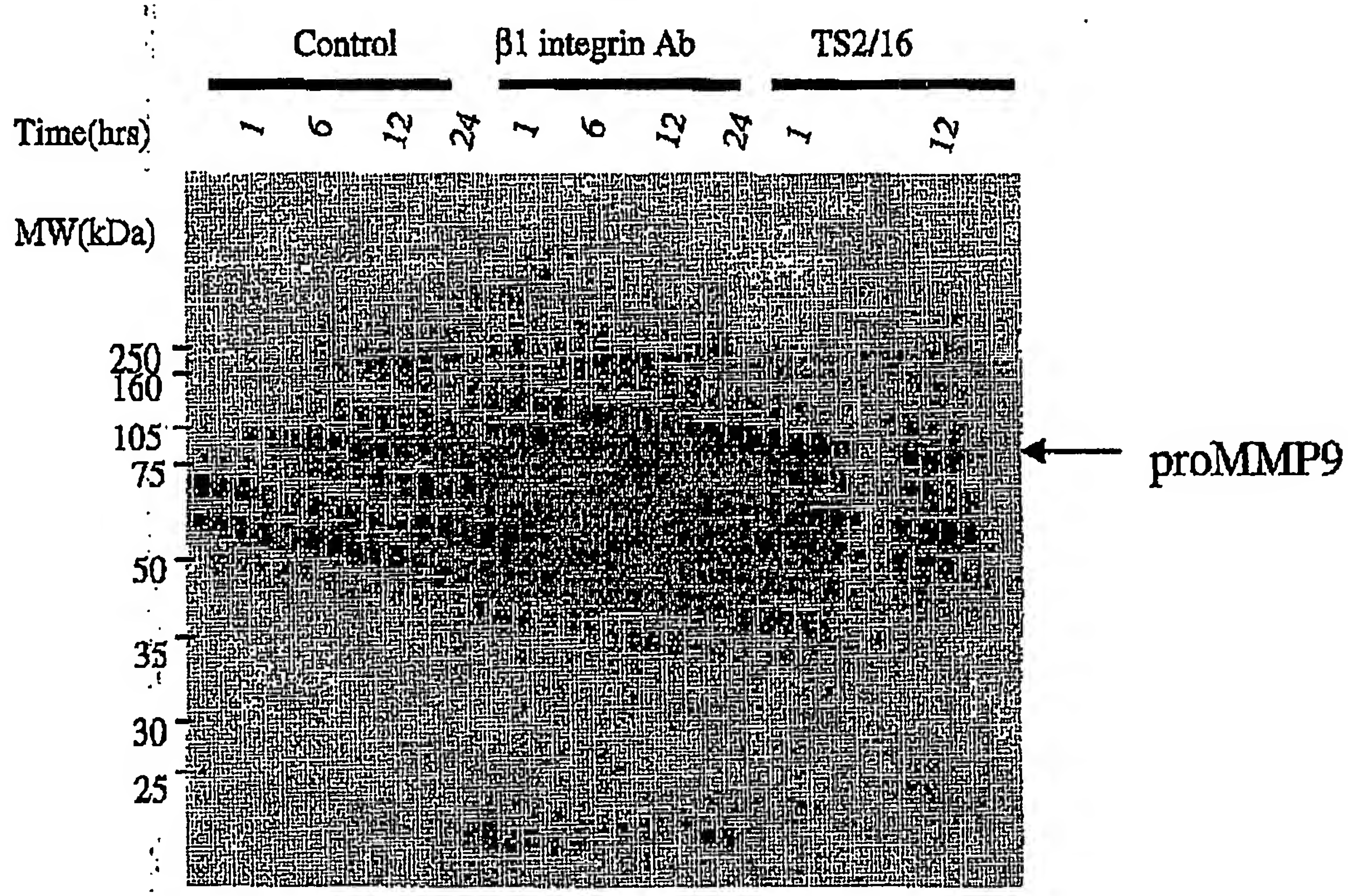


Figure 6. The effect of β1 integrin functional modification on perlecan in cultured human lung cells

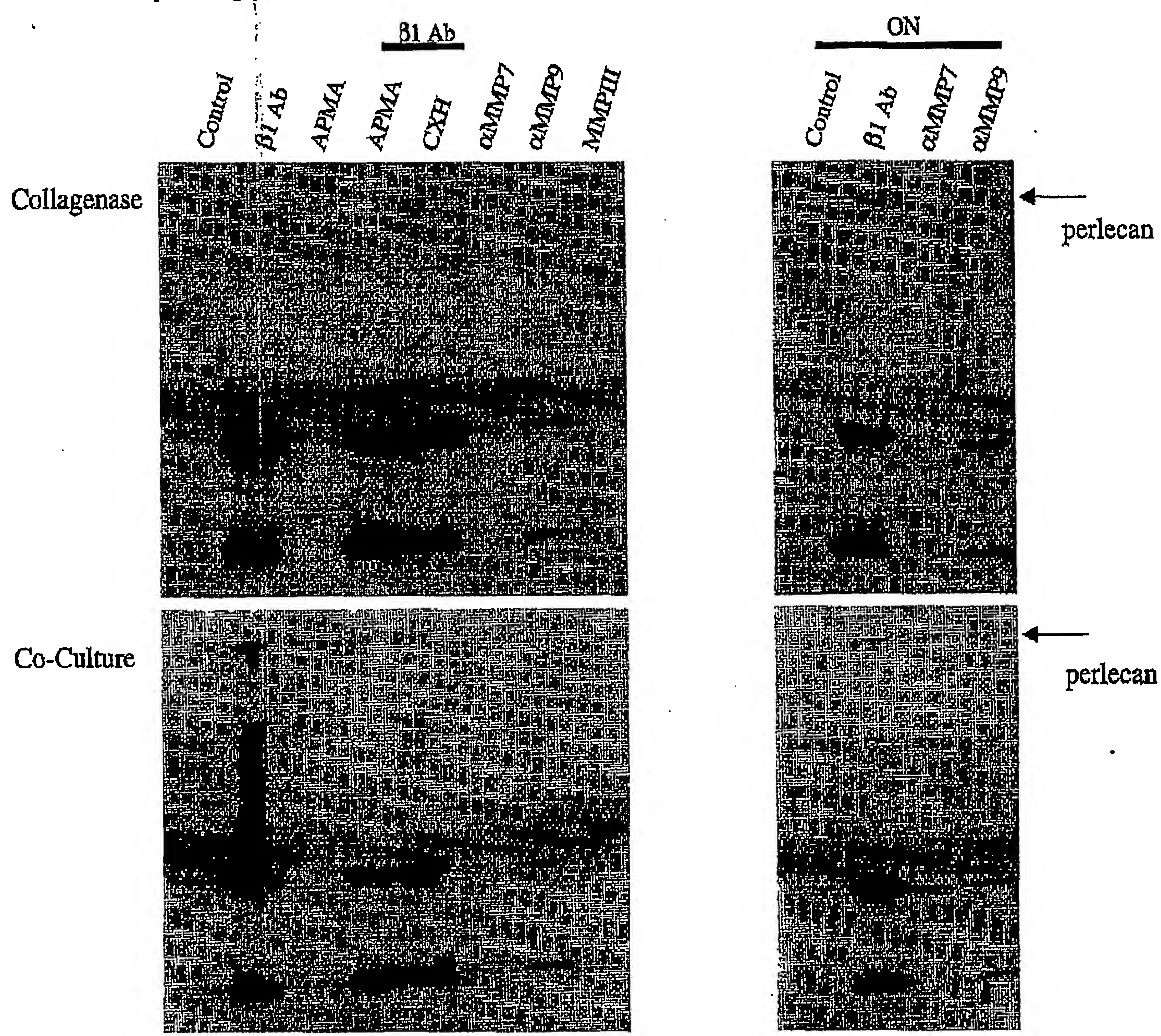


Figure 7. The effect of β1 integrin functional modification on TIMP1 in cultured human lung cells

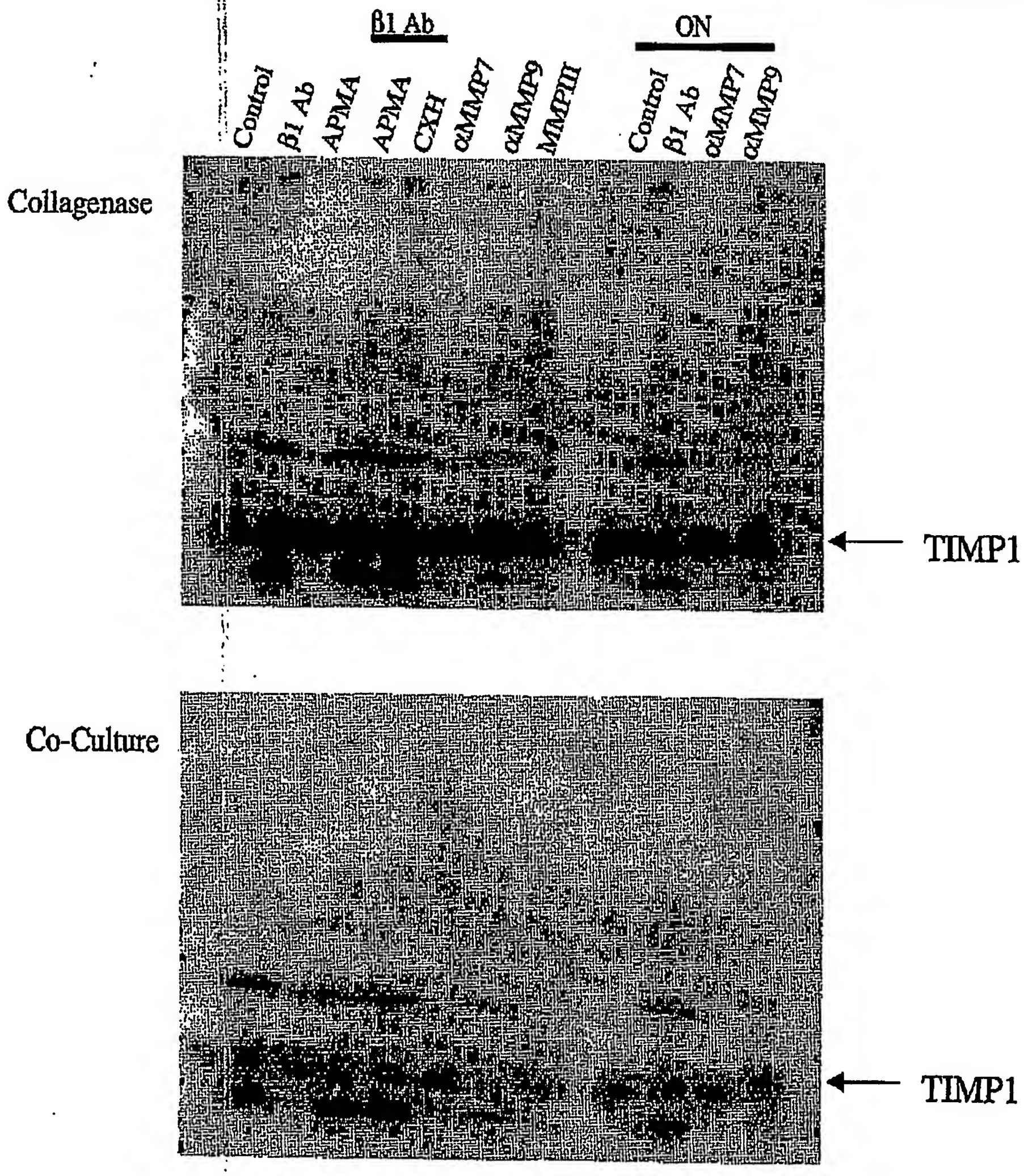


Figure 8. The effect of β1 integrin functional modification on MMP1 in cultured human lung cells

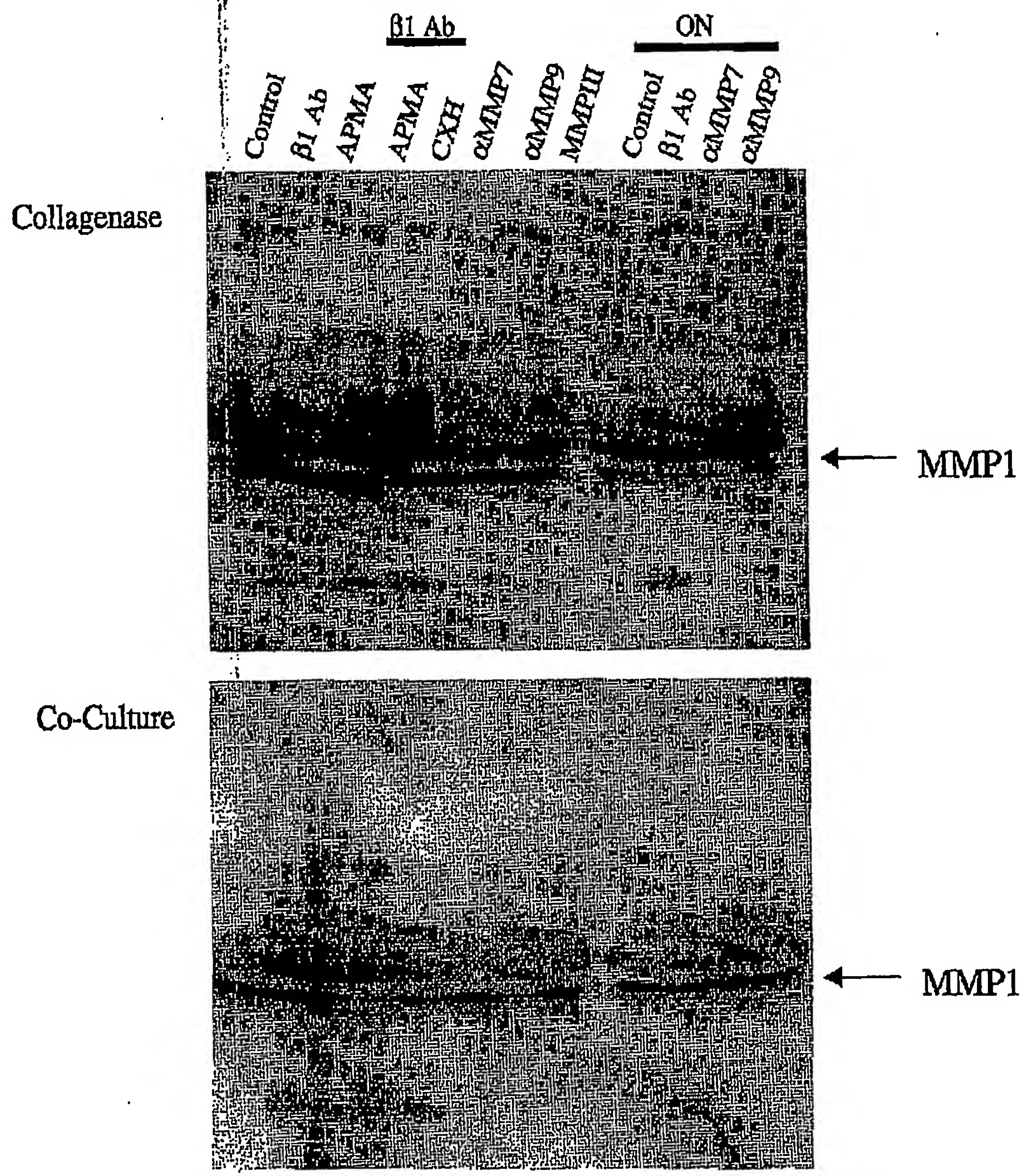


Figure 9. The effect of β1 integrin functional modification on MMP9 in cultured human lung cells

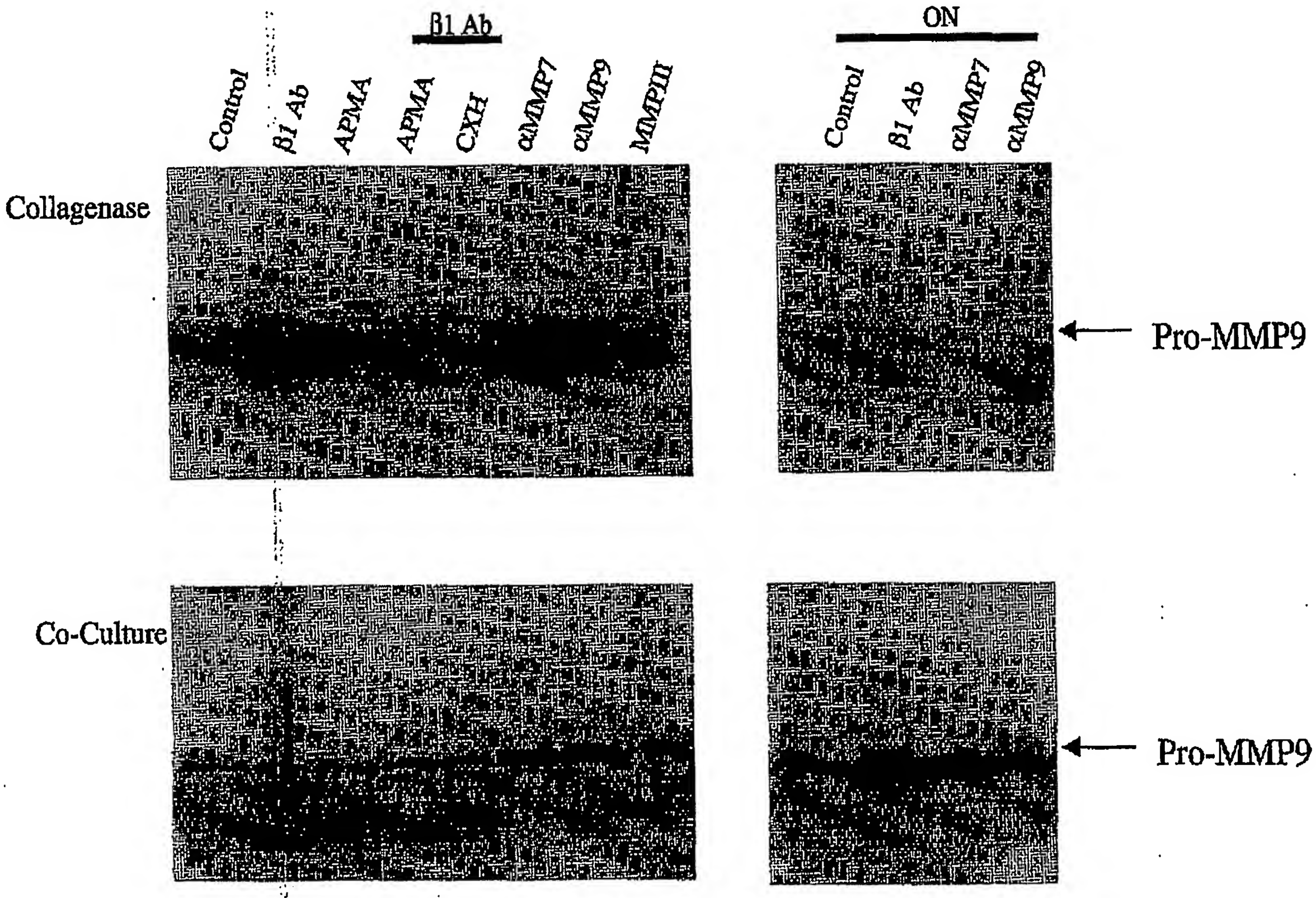
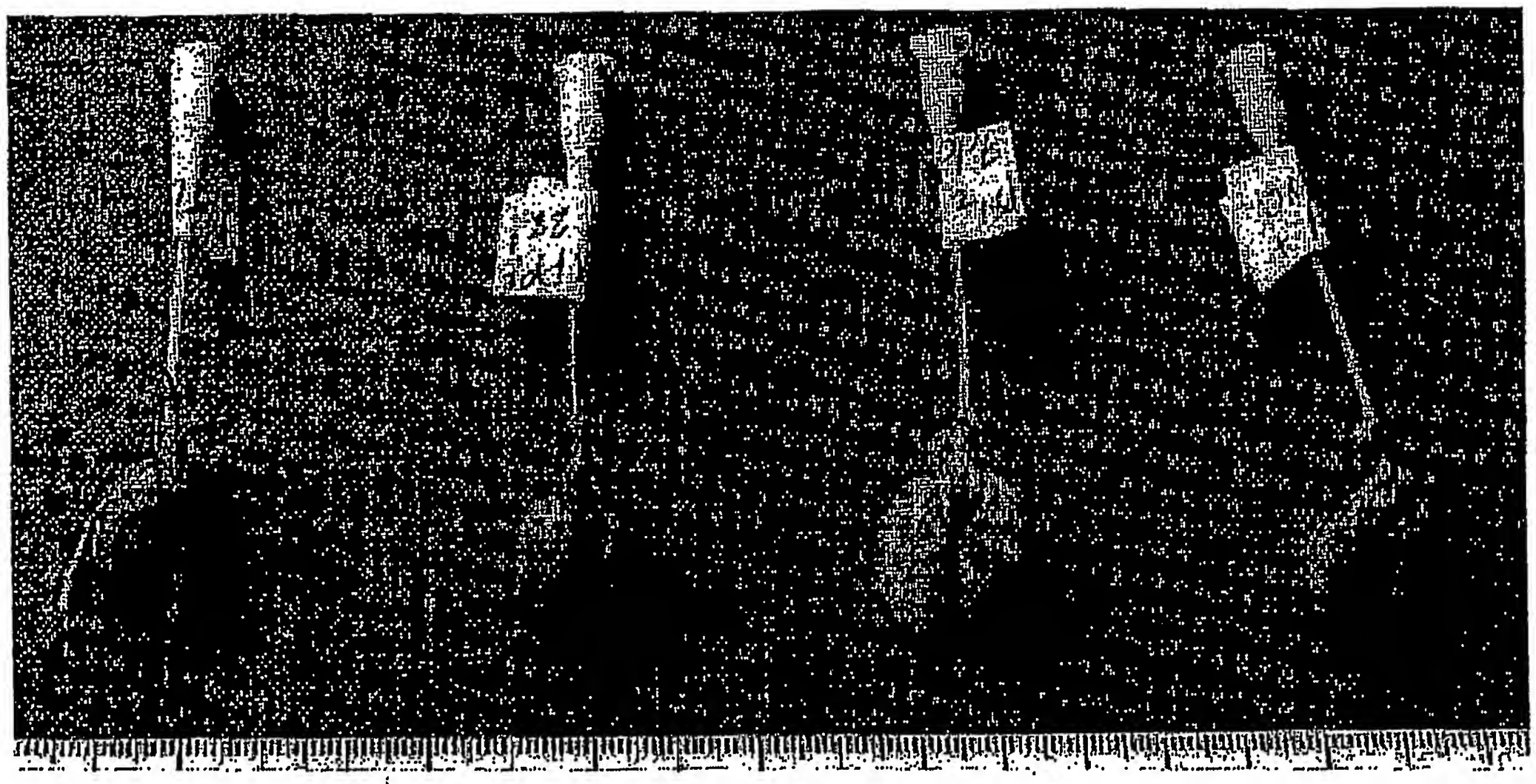


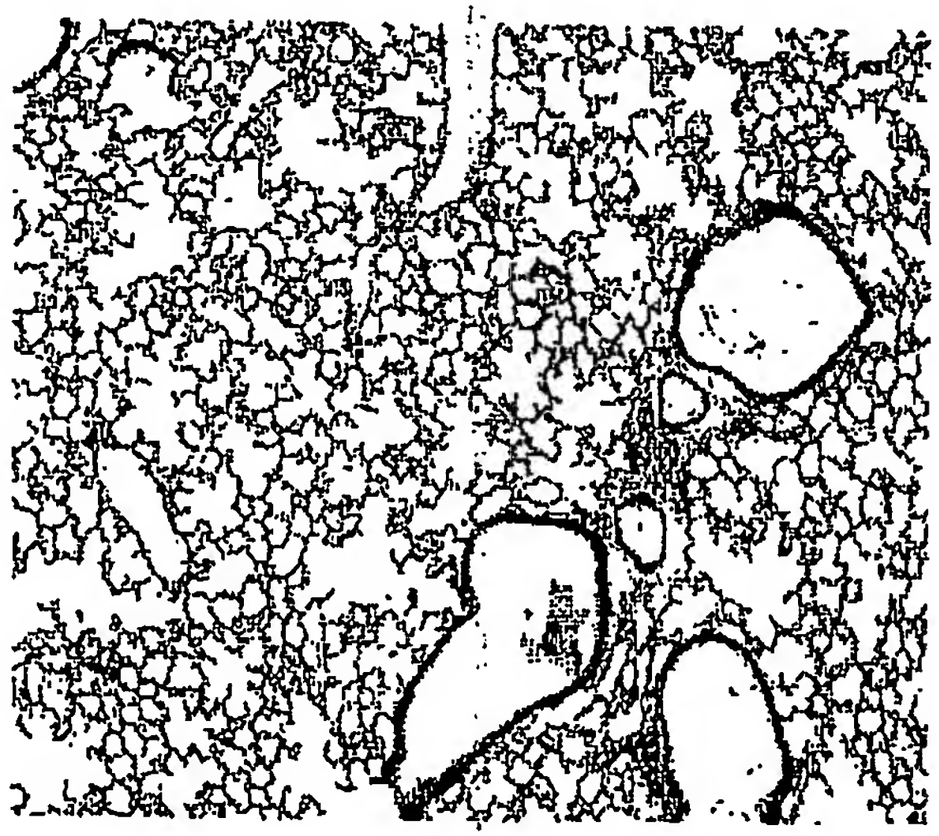
Figure 10. The effect of b1 integrin modulation on emphysematous lungs



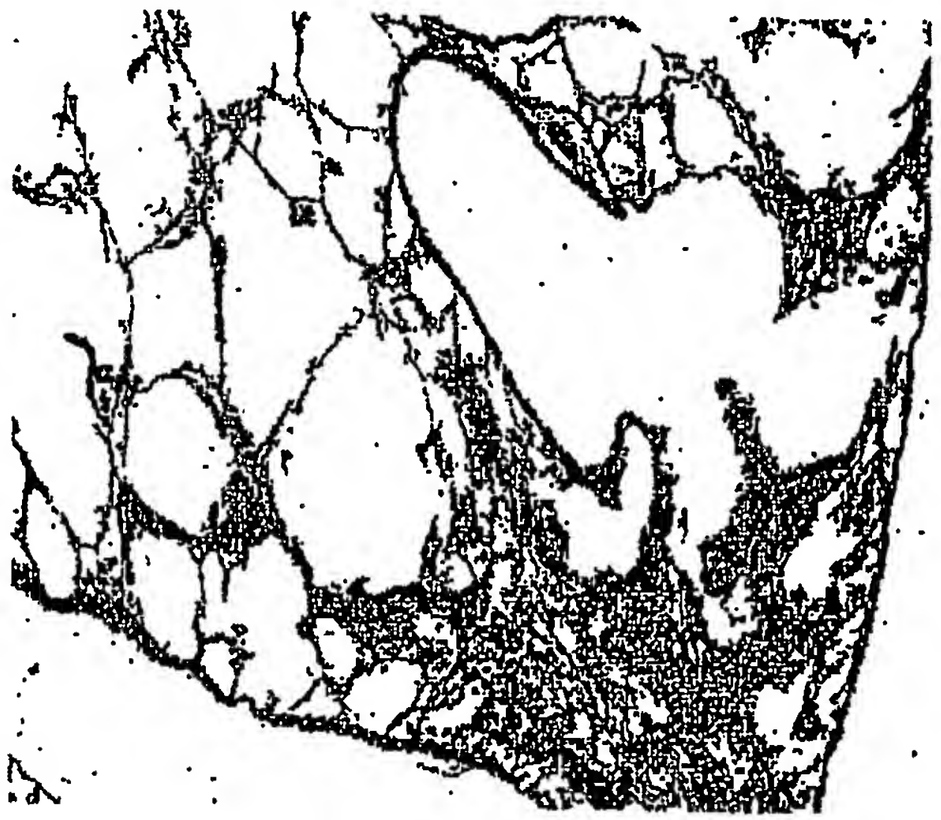
Control **3.5 U PPE** **19.5 U PPE** **19.5 U PPE + anti β1 integrin**

Figure 11.

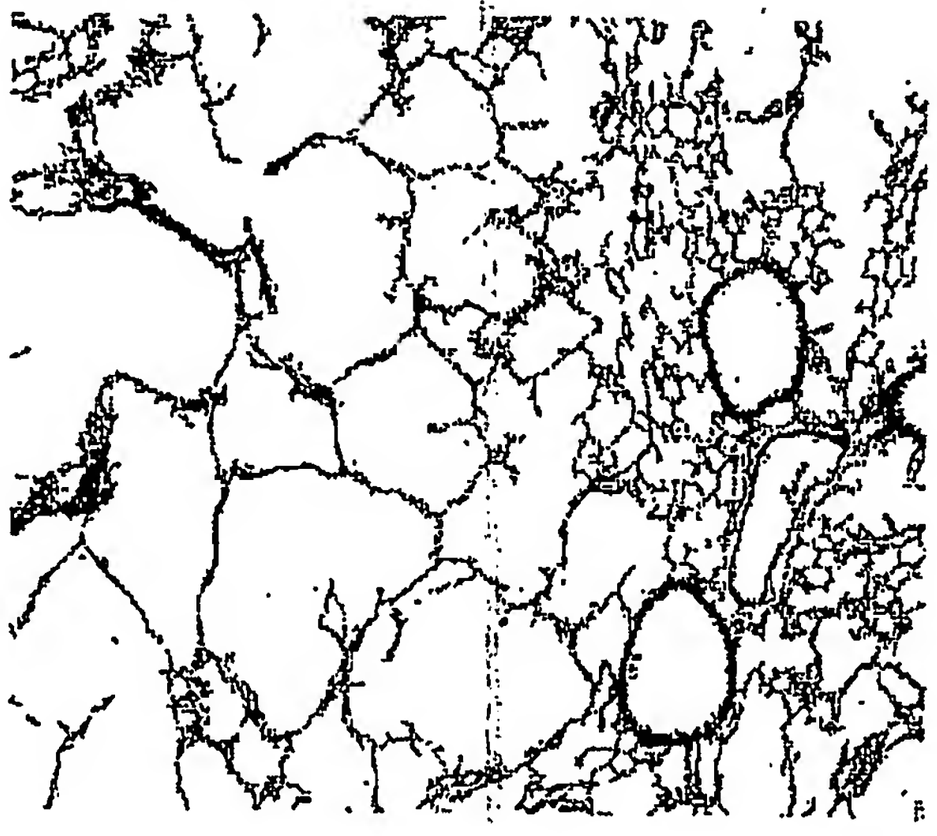
Control



PPE 19.5 U (10d)



PPE 19.5 U (19d)



PPE + anti β1 integrin

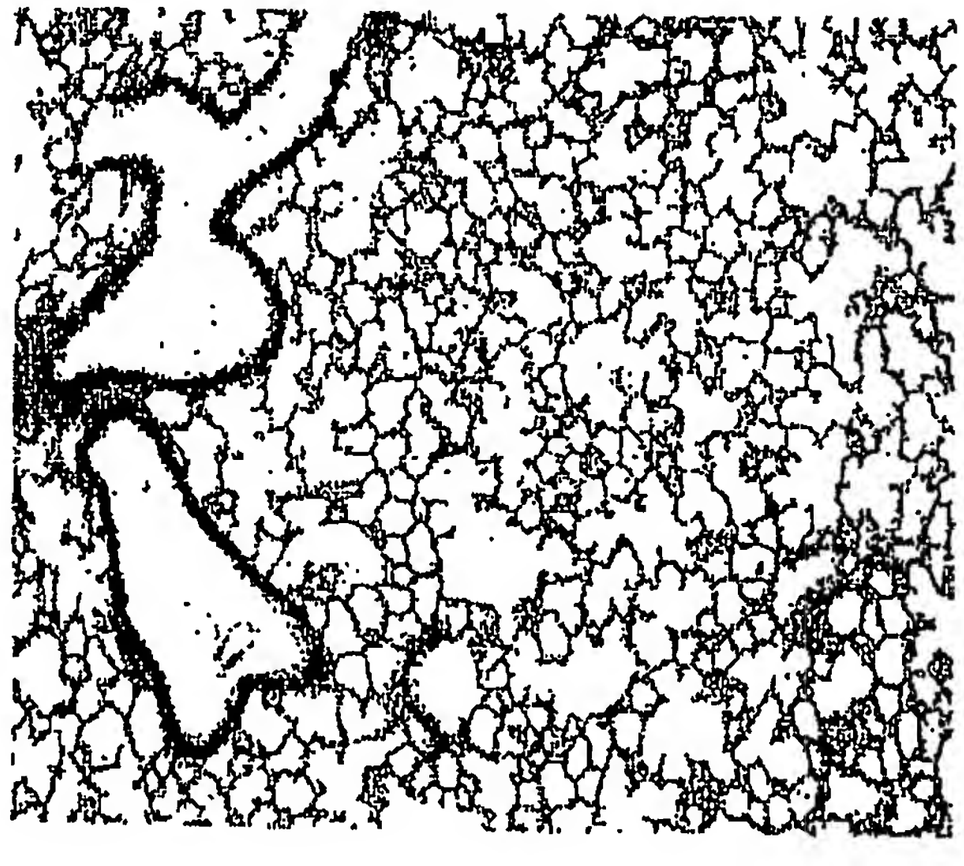


Figure 12. The effect of beta 1 integrin antibody on a space enlargement in elastase-induced emphysema in mice

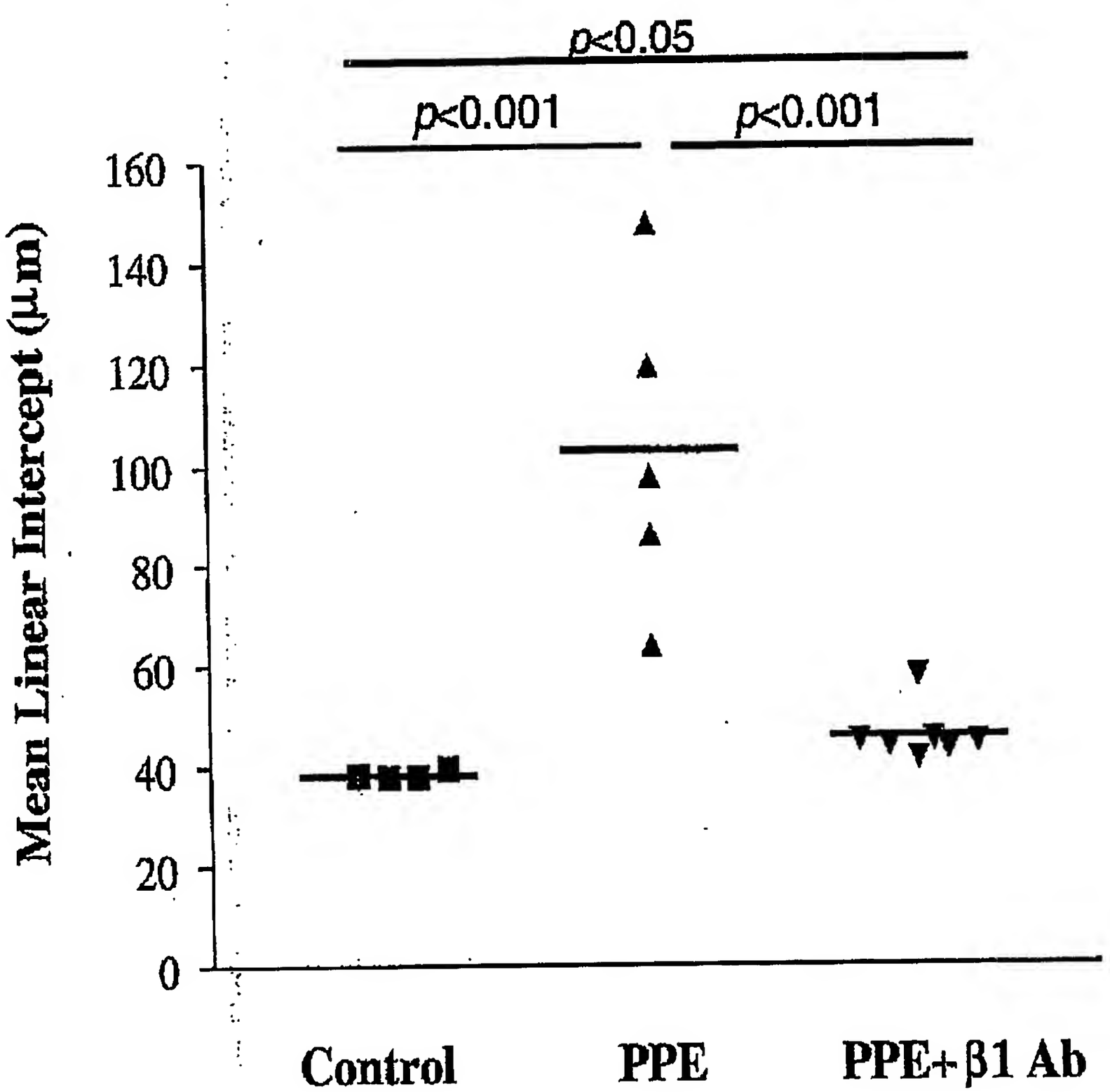


Figure 13. **The effect of β1 integrin antibody on TGFβ1 levels in BAL fluid in elastase-induced emphysema in mice**

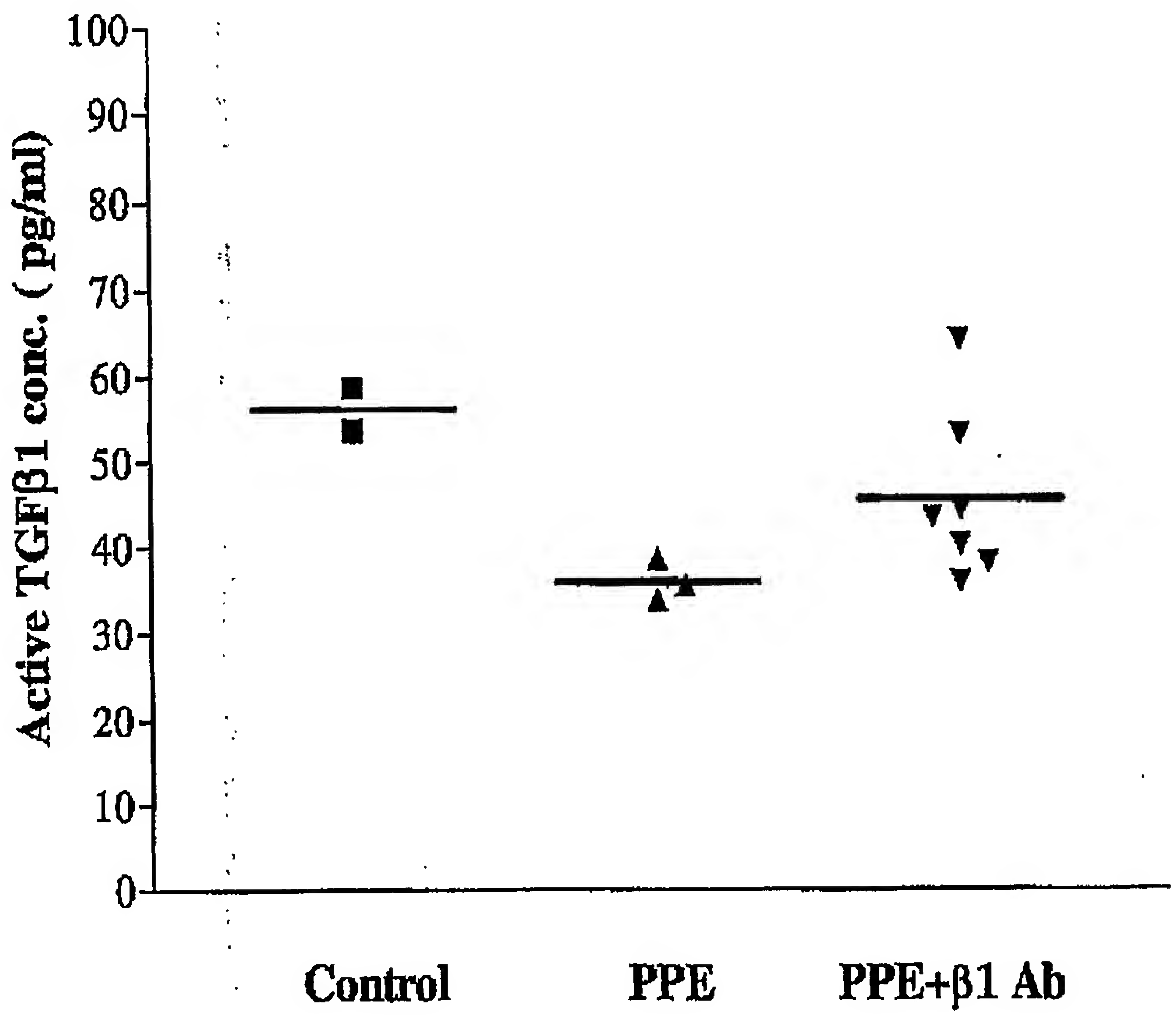


Figure 14. The relationship between airspace enlargement and TGF beta 1 levels in BAL fluid in mice

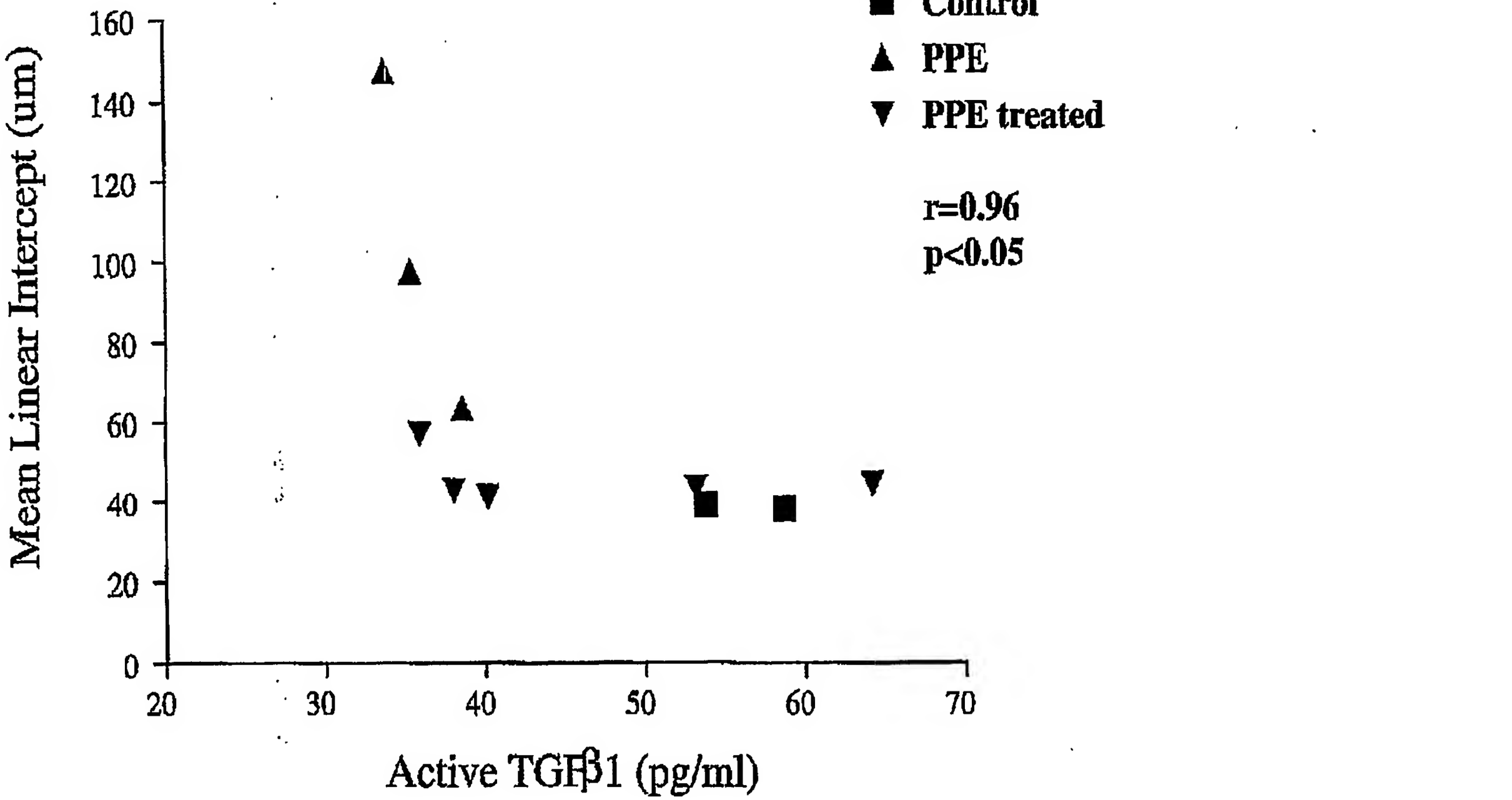


Figure 15. The effect of β1 integrin functional modification on perlecan in NCI-H441 human lung epithelial cell line

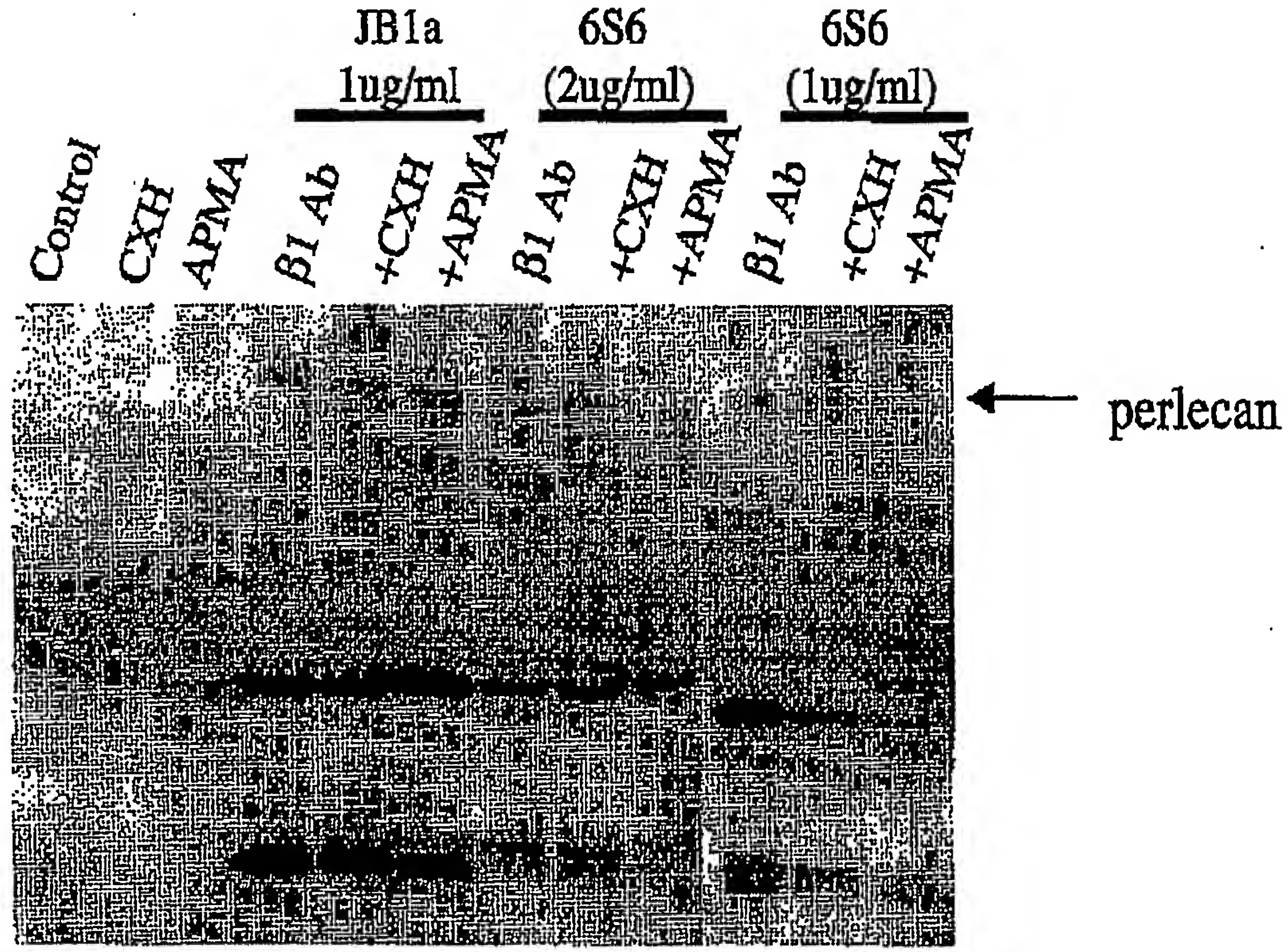


Figure 16. The effect of β1 integrin functional modification on inactive MMP9 in NCI-H441 human lung epithelial cell line